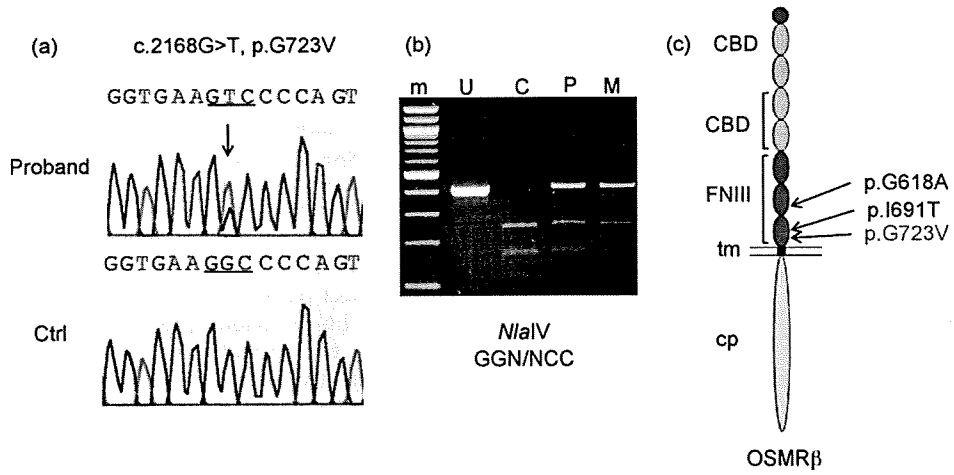

Fig. 2. FPLCA in this family results from the mutation p.G723V in the oncostatin M receptor β. (a) A heterozygous nucleotide substitution, c.2168G > T (NM_003999) in the *OSMR* gene is present in the proband and her mother. (b) The mutation abolishes a restriction enzyme cut site for *Nla*IV, resulting in an undigested DNA band in affected individuals. M, marker; U, control (undigested); C, control (digested); P, proband; M, proband's mother. (Digested PCR product spans exon 15 and flanking introns of *OSMR*.) (c) The mutation p.G723V is located within the FNIII-like domain region, similarly to the previous reported mutations, p.G618A and p.I691T. CBD, cytokine binding domain; FNIII, fibronectin type III-like domain; tm, transmembranous domain; cp, cytoplasmic domain.

OSMRβ were upregulated in psoriatic and atopic skin, suggesting that OSM is a potent activator of skin inflammation [7]. As to PLCA, the histopathological finding of inflammation is less obvious, and main histological features are the pigmentary incontinence and amyloids in the papillary dermis suggesting the apoptosis of basal keratinocytes. Jak/STAT, Erk1/2 and PI3K/Akt signaling, which is downstream of IL-6 type cytokine signaling, have been reported to have antiapoptotic effects in several tumor cell lines [10,11]. These findings suggest the functional decrease of IL-6 type cytokine receptor (including OSMRβ) may lead to a condition that is more susceptible to apoptosis. Recently, diminished innervations of epidermis and dermoepidermal junction were identified in PLCA skin, indicating the damage to the nerve fibers as a possible explanation for the severe pruritis [12]. It is speculated that the pruritis may be the result of hypersensitivity of the remaining nerve fibers as a response to the neurodegeneration [12]. OSMRβ is also expressed in afferent nerve fibers in the spinal cord and the dermis of the skin [13]; therefore, the pathophysiology of PLCA may involve both cutaneous and neural components.

Although FPLCA is relatively common in Asian countries, our case represents the first *OSMR* mutation to be reported in Japanese population.

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Stem Cells, Tissue Engineering and Hematopoietic Elements

Bone Marrow-Derived Cells Are Not the Origin of the Cancer Stem Cells in Ultraviolet-Induced Skin Cancer

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Several lines of evidence have demonstrated that various cancers are derived from cancer stem cells (CSCs), which are thought to originate from either tissue stem or progenitor cells. However, recent studies have suggested that the origin of CSCs could be bone marrow-derived cells (BMDCs); for example, gastric cancer, which follows persistent gastric inflammation, appears to originate from BMDCs. Although our previous research showed the capability of BMDCs to differentiate into epidermal keratinocytes, it has yet to be determined whether skin CSCs originate from BMDCs. To assess the possibility that BMDCs could be the origin of CSCs in skin squamous cell carcinoma (SCC), we used a mouse model of UVB-induced skin SCC. We detected a low percentage of BMDCs in the lesions of epidermal dysplasia (0.59%), SCC *in situ* (0.15%), and SCC (0.03%). Furthermore, we could not find any evidence of clonal BMDC expansion. In SCC lesions, we also found that most of the BMDCs were tumor-infiltrating hematopoietic cells. In addition, BMDCs in the SCC lesions lacked characteristics of epidermal stem cells, including expression of stem cell markers (CD34, high α6 integrin) and the potential retention of BrdU label. These results indicate that BMDCs are not a major source of malignant keratinocytes in UVB-induced SCC. Therefore, we conclude that BMDCs are not the origin of CSCs in UVB-induced SCC. *(Am J Pathol 2009, 174:595–601; DOI: 10.2353/ajpath.2009.080362)*

Stem cells, which have the capacity to self-renew and to differentiate into the various mature cells that constitute the tissue of organ, are found in many adult tissues including the skin.[1] Stem cells are critical for replenishing and maintaining the balance of cells (homeostasis) within the tissue and reconstituting tissue damaged during injury. Numerous studies have shown that the specific stem cell properties and the characteristics of stem-cell systems (populations of cells that derive from stem cells are organized in a hierarchical manner) are relevant to some forms of human cancer.[2,3] In cancers, cancer stem cells (CSCs) are thought to exist. CSCs, like tissue stem cells, would have a capacity for self-renewal and a proliferative ability with successive expansion potential promoting tumor structure organization Tumor-initiating cells, which are considered to be a population rich in CSCs, have been identified in cancers of the hematopoietic system[4,5] and various organs.[6–10]

Although several lines of evidence indicate that CSCs can arise from tissue stem cells[6,8,11,12] or mutated progenitor cells[13,14] current reports showed that gastric cancer, which follows persistent gastric inflammation because of the infection with *Helicobacter felis* (*H. felis*), appears to originate from bone marrow-derived cells (BMDCs).[15] Indeed, some populations of BMDCs have the potential to differentiate into mature cells of various nonhematopoietic organs including liver,[16] skeletal-muscle,[17] brain,[18] and skin.[19] We also showed that BMDCs and mesenchymal stem cells are able to transdifferentiate into keratinocytes.[20,21] BMDCs with this plasticity are frequently recruited to sites of injured or inflamed tissue, where they differentiate into mature tissue cells to contribute to tissue repair.[22] Results from *H. felis*-induced gastric cancer suggest that BMDCs with plasticity would differentiate into tissue stem or mature cells to reconstitute the damaged tissue, they then covert into CSCs, and contribute to carcinoma formation. Although recent investigations have demonstrated that BMDCs could contribute to cancers of small intestine, colon, lung,[23] larynx, and brain,[24] it is yet to be determined whether cancers originating from BMDCs certainly exist.

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Skin cancer is currently the most common malignancy in humans.[25] The skin has the role to protect our bodies from a wide range of environmental assaults including UVB irradiation, chemical carcinogens, and the entry of viruses and other pathogens. Therefore, epidermal keratinocytes have more opportunity to manifest maturation arrest. Particular epidemiological and scientific evidence has shown that UVB is one of the most important factors affecting skin carcinogenesis in the physical environment.[25,26]

As in the case of BMDC-originated gastric cancer after persistent inflammation with *H. felis* infection, it is presumed that BMDCs, which are recruited to the UVB-damaged epidermis and differentiate into epidermal keratinocytes to reconstitute the damaged skin, could then give rise to the maturation arrest during continuous UVB irradiation, convert into CSCs, and finally propagate to form bone marrow (BM)-derived skin cancer. Such a novel hypothesis, if true, would have profound implications for our present understanding of the pathogenesis of squamous cell carcinoma (SCC).

To investigate the possible role of BMDCs in skin cancer, we used a mouse model of UVB-induced skin SCC and evaluated the number and marker expressions of labeled BMDCs that differentiated into keratinocytes in skin SCC.

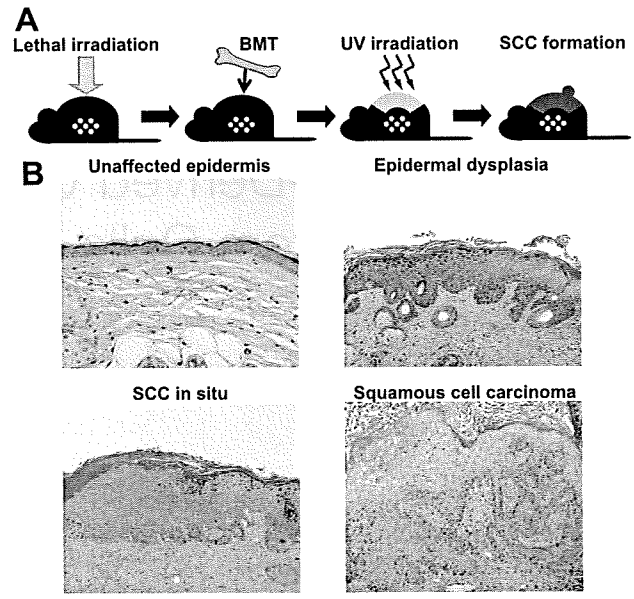

Figure 1. UVB-induced SCC model mice in which BMDCs are labeled with β-Gal enzyme or GFP. **A:** Lethally irradiated mice were transplanted with BM from ROSA26 mice expressing β-Gal enzyme or GFP mice expressing GFP. After confirmation of BM reconstitution, mice were UVB-irradiated. Intermittent UVB irradiation leads mice skin to form SCC. **B:** Tumors were histologically classified as unaffected, dysplasia, SCC *in situ*, or SCC based on tumor architecture, keratinocyte differentiation, and cytological atypia.

Materials and Methods

BM Transplantation

All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol. BM was isolated from the femurs and tibias of male C57BL/6JGtrosa26 (ROSA26) or C57BL/6-TgN(ACTB-EGFP)1Osb/J (GFP) mice (The Jackson Laboratory, Bar Harbor, ME). After lethal irradiation (9 Gy), 1×10^6 BM cells from donor mice in a volume of 200 μl of sterile phosphate-buffered saline were transplanted to recipient C57BL/6 female mice via a single tail vein injection. Hematopoietic reconstitution was subsequently evaluated in peripheral blood 4 weeks after transplantation and more than 94% of BM cells were donor-derived cells.

Induction of UVB Radiation-Induced SCC

UVB-induced carcinogenesis was performed as previously reported (Figure 1A).[27] The UVB light source was a FL20SE30 fluorescent lamp (Clinical Supply, Tokyo, Japan). The UVB irradiation (180 mJ/cm^2) was continued daily for 10 days for tumor initiation to mice (n = 20). One week after the initiation, UVB exposure (180 mJ/cm^2) was performed twice a week until the end of the experiment at 10 months from the last UVB exposure. At 5 months, all irradiated mice (n = 8) had small papules (at least two papules) and erosion. At 10 months, all irradiated mice (n = 6) had tumors (at least three tumors), papules (at least five papules), and ulcer.

Histological Analysis

Mice were sacrificed and tissue was removed, embedded in OCT compound (Sakura, Torrance, CA), snap-frozen or fixed in 4% formalin, and embedded in paraffin. Tumor sections were visualized by routine staining with hematoxylin and eosin (H&E). All of the slides were reviewed twice in blinded manner by three dermatologists, and assessed for tumor architecture, keratinocyte differentiation, cytological atypia, and inflammation. Tumors were classified as dysplasia (typical papilloma), SCC *in situ*, or SCC based on tumor architecture and cytological atypia as described previously.[28] Some lesions exhibiting nonpapillomatous architecture and comprising one to three layers with well-differentiated keratinocytes were classified as normal. Ten samples were analyzed in each normal growth, dysplasia, SCC *in situ*, and SCC. Counts were averaged from eight or nine separate fields in each histological category.

Determination of Enzyme (X-Gal) Activity

Frozen sections (5 μm) were fixed for 30 minutes in 0.2% glutaraldehyde, washed in sodium phosphate buffer containing 0.01% sodium deoxycholate and 0.02% Nonidet P-40 and 1 mmol/L MgCl and incubated for 10 hours at 37°C in a 1-mg/ml X-Gal solution [5-bromo-4-chloro-3-indolyl-β-galactopyranoside: X-Gal, dissolved in dimethyl sulfoxide, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$ $3H_2O$ in 0.1mol/L sodium phosphate buffer] and counterstained with H&E.

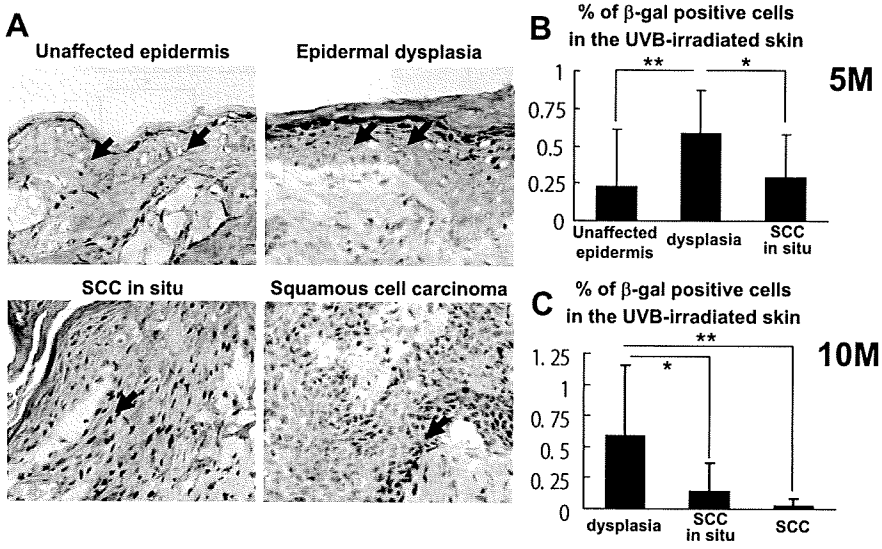

Figure 2. BMDCs in UVB-irradiated mouse skin. **A:** X-Gal-positive cells located within the basal layer in the unaffected epidermis lesions. In the epidermal dysplasia lesions, most X-Gal-positive cells (**arrows**) were found in the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found at the inner part of the tumor. In the SCC lesions, X-Gal-positive cells were also found at the inner part of the tumor. **B:** After 5 months of UVB irradiation, the percentage of X-Gal-positive cells was found at 0.15 ± 0.21% in the unaffected epidermis lesions, increased to 0.58 ± 0.25% in the epidermal dysplasia lesions, and decreased to 0.25 ± 0.20% in the SCC *in situ* lesions (*$P < 0.05$, **$P < 0.01$). **C:** After 10 months of UVB irradiation, the percentage of X-Gal-positive cells was 0.59 ± 0.57% in the epidermal dysplasia lesions and 0.15 ± 0.22% in the SCC *in situ* lesions. In the SCC lesions, the percentage of X-Gal-positive cells in the tumor was decreased to 0.03 ± 0.06% (*$P <$ 0.05, **$P < 0.01$).

Immunofluorescence

Frozen blocks were prepared and sectioned as described above. Sections were fixed in 4% paraformaldehyde and analyzed for β-galactosidase-expressing cells by using polyclonal antibodies (Cappel, Aurora, OH) and fluorescent secondary antibodies (fluorescein isothiocyanate-labeled goat anti-rabbit antibody; Jackson ImmunoResearch, West Grove, PA). Sections fixed in 4% paraformaldehyde were also analyzed for GFP-expressing cells by using polyclonal antibodies (Molecular Probes, Carlsbad, CA). β-Galactosidase-expressing cells were also stained with antibodies to CD45 (BD Biosciences, San Diego, CA), pan cytokeratins (Progen, Heidelberg, Germany), CD34 (BD Biosciences), or α6 integrin (BD Biosciences). Sections ware viewed with a confocal laser-scanning fluorescence microscope (FV1000; Olympus, Tokyo, Japan).

BrdU Assay

The procedure for BrdU pulse labeling and the subsequent detection were performed as previously reported.[29] In brief, at the time of 9-month UVB irradiation, the tumor-bearing model mice were fed with water containing BrdU (1 mg/ml) for 10 days. Forty-five days after BrdU labeling, the tissues were removed. Frozen sections were fixed with 4% paraformaldehyde or 70% ethanol, stained with antibodies to BrdU (Roche, Penzberg, Germany) and fluorescent second antibodies (tetramethyl-rhodamine isothiocyanate-labeled goat anti-mouse antibody; Southern Biotechnology, Birmingham, AL).

Fluorescence in Situ *Hybridization*

X and Y chromosomes were detected on sections from the UVB-irradiated mice skin using a dual-color detection kit (Cambio, Cambridge, UK) according to the manufacturer's protocol (Cy5 for Y chromosomes and Cy3 for X chromosomes) and immediately viewed with a confocal microscope.

Results

Low Frequency of BMDCs in UVB-Irradiated Skin

To investigate the possible role of BMDCs in UVB-induced skin dysplasia/carcinoma progression, we used a model mouse whose BMDCs are labeled with β-galactosidase (β-Gal) or green fluorescent protein (GFP). Lethally irradiated mice were transplanted with BM from ROSA26 mice or GFP transgenic mice (Figure 1A). After the confirmation of BM reconstitution, mice were irradiated with UVB and developing tumors in mice skin were evaluated histologically. Each section was divided into four categories of unaffected, dysplasia, SCC *in situ*, and SCC (Figure 1B).[28] After 5 months of UVB irradiation, we found the dysplasia lesions and the SCC *in situ* lesions, whereas we found no SCC lesions in irradiated skin. After 10 months of UVB irradiation, the dysplasia lesions and the SCC *in situ* lesions were found to be continuous with the SCC lesions, whereas the unaffected epidermis lesions were completely absent.

To detect the presence of BMDCs in UVB-irradiated mouse skin, X-galactosidase (X-Gal) staining was performed. The numbers of BMDCs were quantified by counting the number of X-Gal-positive cells in the UVB-irradiated mouse skin (Figure 2A). After 5 months of UVB irradiation, even in the unaffected epidermis lesions, some X-Gal-positive cells, indicating BMDCs, were located within the basal layer. In the epidermal dysplasia lesions, some X-Gal-positive cells were also found within the basal layer, but most X-Gal-positive cells were found within the suprabasal layers. In the SCC *in situ* lesions, X-Gal-positive cells were found within the inner parts of the tumor. The percentage of occurrence of X-Gal-positive cells was 0.15% in the unaffected epidermis lesions. Since we previously showed that wounded skin contained BMDCs (0.03%),[20] repeated UVB irradiation might induce BMDC accumulation. The percentage of X-Gal-positive cells in the epidermal dysplasia lesions increased to 0.58%, whereas the percentage of X-Gal-

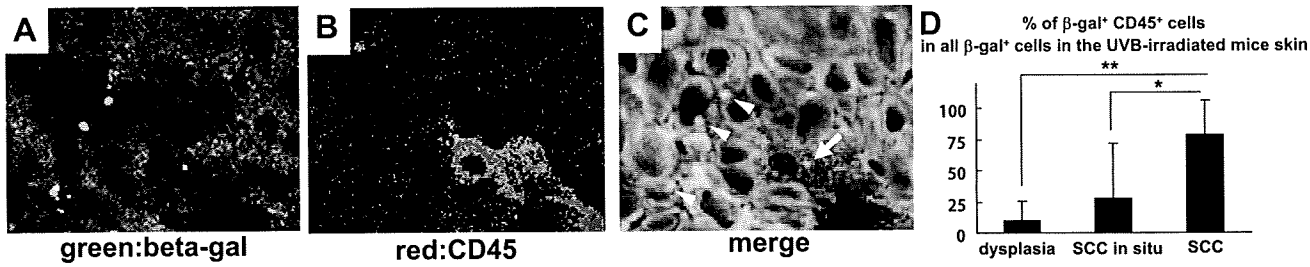

Figure 3. BM-derived infiltrating cells in the UVB-irradiated skin. Triple staining of β-Gal (green) (**A**), CD45 (red) (**B**), and pancytokeratin (cyan) was performed. **C:** Merged image showed β-Gal$^+$/CD45$^+$ (**arrow**) or β-Gal$^+$/CD45$^-$ (**arrowheads**) cells in the tumor. **D:** Percentage of CD45$^+$ of all β-Gal$^+$ cells in the UVB-irradiated mice skin. In all β-Gal$^+$ cells, 10.1 ± 15.3% was positive for CD45 in the epidermal dysplasia lesions, 27.3 ± 44.1% in the SCC *in situ* lesions, and at 78.7 ± 27.4% in the SCC lesions ($^*P < 0.05$, $^{**}P < 0.01$). Original magnifications, ×600.

positive cells in the SCC *in situ* lesions was decreased to 0.25% (Figure 2B). The number of β-Gal-positive cells and total epidermal cells of the UVB-irradiated skin were as follow; unaffected (6 of 2276), dysplasia (28 of 4804), SCC *in situ* (15 of 5445). We further confirmed that no X-Gal-positive cells were detected in untreated (unirradiated) mice. We failed to find any clusters of X-Gal-positive cells in either the unaffected epidermis or the tumor. These results indicate that BMDCs in the UVB-irradiated skin do not commonly give rise to a monoclonal expansion.

After 10 months of UVB irradiation, in the epidermal dysplasia lesions and SCC *in situ* lesions, we found X-Gal-positive cells in a similar location as mice skin that received 5 months of UVB irradiation. In the SCC lesions, X-Gal-positive cells were found within the inner part of the tumor (Figure 2A). X-Gal-positive cells were found at a percentage of 0.59% in the epidermal dysplasia lesions and 0.15% in the SCC *in situ* lesions. These percentages of X-Gal-positive cells in 10-month UVB-irradiated mouse skin were similar to the percentage in 5-month UVB-irradiated mouse skin. In the SCC lesions, the percentage of X-Gal-positive cells was at 0.03%, which decreased in comparison with the percentage in the SCC *in situ* lesions (Figure 2C). The number of β-Gal-positive cells and total epidermal cells of the UVB-irradiated skin were as follow; dysplasia (28 of 5141), SCC *in situ* (9 of 6559), SCC (4 of 13,701).

As an additional test for BM origin, we used a mouse model in which BMDCs were GFP$^+$ using BMT from GFP transgenic mice. Although we evaluated the percentages of BMDCs in UVB-irradiated skin, the GFP$^+$/pancytokeratin$^+$ cells were found at an extremely low percentage, ~0.12% in the epidermal dysplasia lesions and 0% in the SCC *in situ* lesions (data not shown). Previous reports about the *H. felis* gastric cancer also showed a similar tendency that the percentages of malignant cells with the marker of BMDCs was much lower in GFP-labeled model mice than in β-Gal-labeled model mice.[15] Therefore we used an UVB-irradiated mouse model with labeled BMDCs with β-Gal in the following experiments.

Most BMDCs in the SCC Are Inflammatory Hematopoietic Cells

We considered that some X-Gal-positive cells in the UVB-irradiated skin were likely to be the tumor-infiltrating hematopoietic cells. To investigate the presence of these cells, triple staining for β-Gal, CD45 (hematopoietic marker), and a pancytokeratin (cytokeratin marker) was performed (Figure 3, A–C). The number of β-Gal$^+$/CD45$^+$ of all β-Gal$^+$ cells per field was counted in UVB-irradiated mouse skin. In all β-Gal$^+$ cells, 10.1% were positive for CD45 in the epidermal dysplasia lesions. Percentages of CD45$^+$ cells of all β-Gal$^+$ cells were 27.3% and 78.7% in the SCC *in situ* lesions and in the SCC lesions, respectively (Figure 3D). Some of the CD45$^+$ cells were fused with carcinoma cells. Indeed, CD45 has been found to be expressed by cancer cells.[30–32] However, we were unable to find X-Gal-positive cells that co-expressed CD45 and pancytokeratin. The result of our experiments clearly shows that some β-Gal$^+$ cells are tumor-infiltrating hematopoietic cells, whereas other β-Gal$^+$/CD45$^-$ cells might be BMDCs that differentiated into tumor keratinocytes. However, the percentage of β-Gal$^+$/CD45$^+$ cells (indicating tumor-infiltrating hematopoietic cells) is increased in the SCC lesions. This observation would indicate that the actual occurrence rate of BM-derived keratinocytes is lower than our counting of BMDCs that were detected with X-Gal staining.

Small Number of BMDCs in the SCC Exhibited Donor XY Chromosomes

To further confirm BM origin, we analyzed UVB-induced skin SCC cells from female hosts (XX chromosomes) transplanted with male donor BM (XY chromosomes) using fluorescence *in situ* hybridization technique. We counted more than 10,000 cells and detected some donor-derived keratinocytes with XY chromosome expression, indicating BM origin (less than 0.05%) (Figure 4A).

In various organs, BMDCs contribute to the tissue reconstitution by either fusion[22] or transdifferentiation.[19] To determine whether BMDC engraftment into the specific tissue cells was because of differentiation or somatic cell fusion, fluorescence *in situ* hybridization was used because the fused cells would be expected to possess XXXY chromosomes. Although we observed keratinocytes with Y chromosomes in the tumor, none of them expressed an XXXY chromosome. However, fusion hybrids notoriously lose chromosomes and the absence of tetraploid cells does not rule out fusion.[33–35] Therefore, we could not exclude the possibility of cell fusion with the present data.

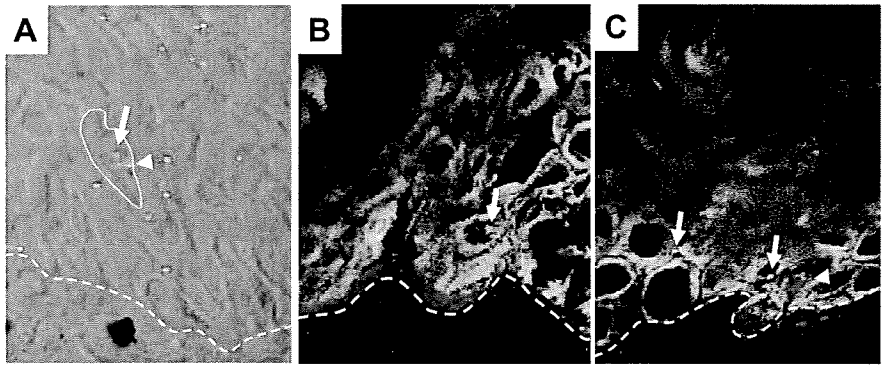

Figure 4. XY chromosome expressions and epidermal stem cell markers of the BMDCs in the UVB-irradiated skin. **A:** Fluorescence *in situ* hybridization showed cells with single X chromosome (red, **arrow**) and single Y chromosome (cyan, **arrowhead**) in the UVB-induced skin SCC. XY chromosome cells, indicating BMDCs were indicated. **B:** Triple staining of β-Gal (green), α6 integrin (red), and pancytokeratin (cyan) was performed. **Arrow** shows β-Gal$^+$ tumor keratinocytes. Although α6 integrin was positive within the edge of the tumor, we could not find any significant overexpression of α6 integrin of β-Gal$^+$/cytokeratin$^+$ cells. **C:** Triple staining of β-Gal (green), BrdU (red), pancytokeratin (cyan). **Arrows** show β-Gal$^+$ tumor keratinocytes. **Arrowhead** shows a BrdU$^+$ tumor keratinocyte. We found no β-Gal$^+$/BrdU$^+$ tumor keratinocytes.

BMDCs in the SCC Failed to Express Epidermal Stem Cell Marker

Although the CSC markers of skin SCC have yet to be defined, published studies suggest that tumor-initiating cells might be positive for the stem cell marker of the original organs.[6,8] To investigate the possibility that BMDCs in the UVB-irradiated skin could share some characteristics of CSCs of skin SCC, we assayed the location of these presumptive CSCs that are positive for epidermal stem cell markers in the UVB-induced skin SCC.

Although CD34 is an established marker of skin epithelial stem cells,[36] none of the keratinocytes (including BM-derived keratinocytes) in the UVB-induced skin SCC expressed CD34 (data not shown). Furthermore, skin epithelial stem cells express elevated levels of α6 integrin compared with differentiated keratinocytes.[37] Although some keratinocytes in the edge of SCC showed α6 integrin expression, β-Gal$^+$/pancytokeratin$^+$ cells (indicating BM-derived keratinocytes) did not show significantly upregulated α6 integrin expression compared with non-BM-derived keratinocytes (Figure 4B). In addition, tissue stem cells can be distinguished from transit-amplifying cells by their ability to incorporate and retain 5-bromo-2′-deoxyuridine (BrdU) throughout a long period of time. Therefore, tissue stem cells can be identified as label-retaining cells (LRCs).[29] To determine whether BMDCs in the UVB-irradiated mouse skin exhibit any LRC characteristics, the tumor-bearing mice were fed water containing BrdU. In the UVB-irradiated mice skin, no LRCs expressed β-Gal (Figure 4C). These results indicate that BMDCs in the UVB-induced skin SCC did not share any of these characters of the presumptive CSCs of the skin SCC.

Discussion

Based on recent investigations that suggest the possibility for BMDCs to be the origin of cancers,[15,38] we used a labeled BMDC mouse model and investigated the role of BMDCs during UVB-induced carcinogenesis. With intermittent UVB irradiation, the epidermal morphology in mouse skin changed from the normal state through dysplasia, SCC *in situ*, and finally to SCC. These histological changes are analogous to the natural phenomenon observed in UVB-induced human skin carcinogenesis. We certainly found BMDCs in UVB-irradiated mouse skin. Our data further suggests that BMDCs are recruited to the UVB-damaged skin and transdifferentiate into epidermal keratinocytes to reconstitute the skin, as we previously reported in wound repair.[20] We show the accelerated recruitment of BMDCs in the epidermal dysplasia lesions and the decreased rate of BMDCs in the SCC lesions. We propose this is attributable to the propagation of non-BM-derived malignant keratinocytes. Although BMDCs are recruited to the UVB-damaged skin and transdifferentiate into unaffected epidermal keratinocytes, BMDCs do not convert into malignant keratinocytes so that the rate of BMDCs relatively decreases as non-BM-derived tumor keratinocytes propagate to form skin SCC.

As a result, we found very few instances of BM-derived keratinocytes in the UVB-irradiated mouse skin. This observation strongly suggests that BMDCs are unlikely to be the origin of UVB-induced skin SCC. The objection will no doubt be raised that BMDCs might lose the expression of BM markers during the continuous UVB irradiation. Therefore we were careful to examine BM-derived keratinocytes in skin SCC with three different BMDC markers (β-Gal, GFP, Y chromosome analysis). Our conclusion is exactly the opposite of the *H. felis*-induced murine gastric carcinoma study.[15] It is reasonable to suppose that the difference in the results between *H. felis*-induced gastric carcinoma study and our UVB-induced skin carcinoma study is partially attributable to the process of carcinogenesis including the type of genetic damage and degree of inflammation. In *H. felis*-induced gastric carcinoma, the pathogenic factor, namely CagA, increases the proliferation of host cells or inhibits cell apoptosis, stimulating the malignant transformation of host cells.[39,40] These processes would be important for cancer progression from BMDCs. In humans, previous reports showed that solid cancers contain BM-derived cancer cells at a low level of 0 to 6% except for lung carcinoma that contains ~20% of BM-derived cancer cells.[23,24] These data further showed that BMDCs do not contribute to skin cancers.[23] Our results are consistent with these observations.

The epidermis is continuously supplied with keratinocytes from the hair follicle bulge stem cells throughout adult life.[41] Most epidermal keratinocytes that acquire

oncogenic mutations are lost during differentiation. Therefore, only long-term resident cells, such as stem cells, have the capacity to accumulate the required number of genetic hits necessary for tumor development. For this reason, it is not unreasonable to assume that these epidermal stem cells in the bulge could acquire oncogenic mutations, transdifferentiate into CSCs, and proliferate as malignant cells in the skin cancer. Although a previous report showed that BMDCs were more frequently found in the bulge area,[42] we could not find such a tendency in our experiments in UVB-induced carcinogenesis. Our previous research in the damaged skin also showed no tendency of BMDC accumulation at specific skin sites.[20] Furthermore, we failed to find any evidence of BMDC clonal expansion in the UVB-irradiated mice skin. We also showed that BMDCs express no epidermal stem cell markers and fail to behave as LRCs, one of the main characteristics of tissue stem cells. Although the existence of the CSCs in the skin cancer has yet to be properly defined, we suggest that the CSCs in the UVB-induced skin SCC, if present, do not commonly originate from BMDCs.

It is important to determine the origin of the CSCs for the elucidation of carcinogenic mechanisms or for the treatment of cancer. Because of the recent reports that showed sarcoma derived from mesenchymal stem cells,[43,44] an objection against transferring cells with the potential to have properties of stem or progenitor cells has arisen in regenerative medicine. However we can conclude from the results of our experiments that cancer cells in the UVB-induced skin SCC do not originate from BMDCs. Therefore we consider that in adopting or using BMDCs for regenerative medicine, the possibility of unexpected carcinogenesis can primarily be excluded and that BMDCs should be further tested and adapted for use in regenerative medicine, especially for skin.

We demonstrated the existence of BM-derived keratinocytes in the UVB-irradiated skin. These BM-derived keratinocytes were considered to be the result of transdifferentiation, not fusion. However, the number of BM-derived keratinocytes was extremely few, with no clonal expansion. Furthermore, BM-derived keratinocytes failed to express the epidermal stem cell markers (CD34, high α6 integrin and LRCs). Through our laboratory experiments, the possibility that BMDCs are the origin of UVB-induced skin SCC is extremely low.

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Conradi–Hünermann–Happle syndrome with abnormal lamellar granule contents

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Sir, Conradi–Hünermann–Happle syndrome (CHH) (X-linked dominant chondrodysplasia punctata type II, MIM 302960) is an X-linked dominant inherited disorder, characterized by linear ichthyosis, chondrodysplasia punctata, cataract and short stature.[1] The gene for this disease has been identified as EBP encoding the emopamil binding protein (EBP), located on the short arm of the X chromosome.[2,3] However, the exact pathomechanisms of how EBP defects cause the CHH disease phenotype have yet to be clarified. Ultrastructural features of CHH epidermis have thus far not been reported in patients whose EBP mutations have been identified. Here, we have ultrastructurally examined the epidermis of a patient with CHH carrying the EBP mutation p.Arg147His and have demonstrated abnormal contents of lamellar granules in the lesional granular keratinocyte layers.

A female newborn with skin scaling and shortened extremities was referred to us. The pregnancy had been uneventful except for excessive amniotic fluid, and the baby was born spontaneously at 37 weeks 4 days gestational age by normal vaginal delivery. The birth weight was 2696 g and the height at birth was 44·0 cm. No respiratory distress was observed at birth. The baby was the second child of nonconsanguineous, healthy parents. There was no other affected member in the family, including the proband's healthy elder brother. Frontal bossing, flat nasal bridge and shortened neck were noted at birth (Fig. 1). The right upper and lower extremities of the patient were shortened. Whole body X-ray examination revealed punctate calcification in the epiphyseal regions of the majority of long bones (Fig. 1), including shoulder joints, elbow joints, wrist joints, hip joints, knee joints and ankle joints, and the calcification was most severe on the right side of her body. Her height was below the third percentile at birth and during all the postnatal period. Her weight was also below the third percentile from 2 months of age, although it was between the 10th and the 25th percentile at birth. She had bilateral anterior polar cataracts, which were more severe on the right side. During the neonatal period, linear and whorled hyperkeratosis was seen on erythrodermic skin and the thick scales were more severe on the right side of her

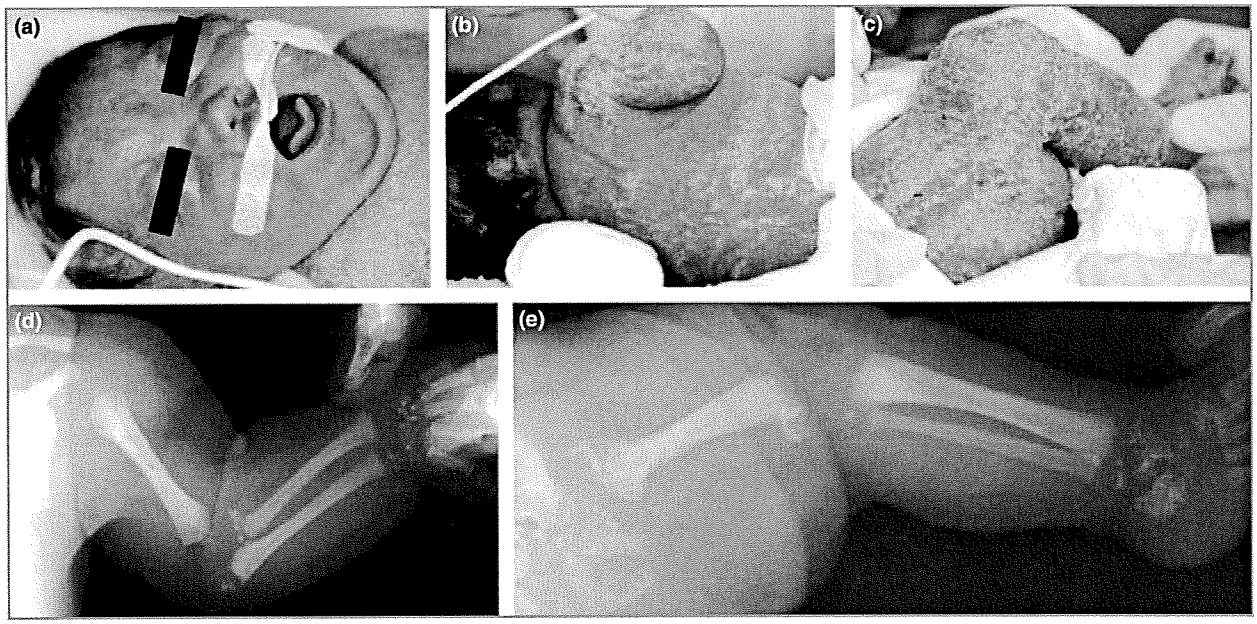

Fig 1. Clinical and X-ray appearance of the patient. (a) Frontal bossing and flat nasal bridge were seen on the face. (b) Circumscribed alopecia was noted on the scalp. (b, c) Linear and whorled hyperkeratosis was seen on a background of erythrodermic skin on the back (b) and over the thigh (c). (d, e) X-ray showed punctate calcification in the epiphyseal growth plate of the bones of the right arm (d) and in the right leg and hip (e).

body (Fig. 1). The entire body surface was erythematous and the extremities were oedematous. Circumscribed alopecia was more pronounced on the right side of the scalp. The skin eruptions cleared within several weeks, although slightly scaly skin remained over her whole body. Skin biopsy of a hyperkeratotic lesion on the trunk taken during the neonatal period revealed orthohyperkeratosis. A marked calcification in the stratum corneum was seen by van Kossa staining. From these clinical and histological features, a diagnosis of CHH was made in this case.

All four coding exons 2–5 of EBP were amplified using previously described polymerase chain reaction (PCR) primers.[4] Direct sequencing of the PCR products from the patient and her parents revealed that the patient was a heterozygote for a missense mutation p.Arg147His [G to A substitution at nucleotide position 440: arginine 147 (CGC) to histidine (CAC)], which was not found in her parents. This mutation was not found in 100 normal unrelated alleles (50 normal unrelated Japanese individuals) by direct sequencing analysis. Direct sequencing of all the coding exons and exon/intron borders of EBP failed to detect any other pathogenic mutations in the patient's DNA. The p.Arg147His is a known mutation reported in an aborted fetus affected with CHH.[3]

We performed ultrastructural observation of the patient's epidermal keratinocytes using ruthenium tetroxide postfixation. Lamellar granules with abnormal contents, lacking the normal lamellar structure, were seen in the granular layer keratinocytes in the patient's epidermis (Fig. 2). The lamellar granule contents were secreted into the intercellular space in the stratum corneum. Secreted lipid material trapped in the cytoplasm of corneocytes, corresponding to the membranous remnants reported by Emami *et al.*,[5] was distributed sparsely throughout the stratum corneum. In addition, irregularly dilated intercellular spaces were often observed between the keratinized cells.

We performed CD1a staining on the skin biopsy specimen in order to evaluate Langerhans cell density in the epidermis. Langerhans cell density was 8·8 cells/high power field (HPF) in the patient's epidermis and 4·2 cells/HPF and 16·0 cells/HPF in two age/gender-matched normal controls. Thus, no significant reduction of Langerhans cell density was confirmed in the patient's epidermis.

EBP mutations were reported to underlie CHH. EBP has a dual function: on the one hand it serves as a binding protein for the Ca^{2+} antagonist emopamil and thus is a high-affinity acceptor protein for several anti-ischaemic drugs,[6] and on the other hand it acts as a delta8–delta7 sterol isomerase.[7] It has been suggested that the skeletal manifestations in CHH may be caused by an accumulation of toxic sterol intermediates which interfere with the function of cholesterol-modified hedgehog proteins.[8] Furthermore, the molecular pathology of the ichthyotic phenotype in CHH can also be explained by EBP function in sterol biosynthesis pathways. EBP is a key enzyme involved in cholesterol biosynthesis[7] and dysfunction of EBP results in cholesterol deficiency and accumulation of cholesterol pathway products such as 8-dehydrocholesterol.[2,3] In a review of the pathophysiology of ichthyosis disease, Elias *et al.*[9] hypothesized that a deficiency of bulk cholesterol accumulation in keratinocyte membrane function may be a major factor contributing to the ichthyosis phenotype seen in CHH.

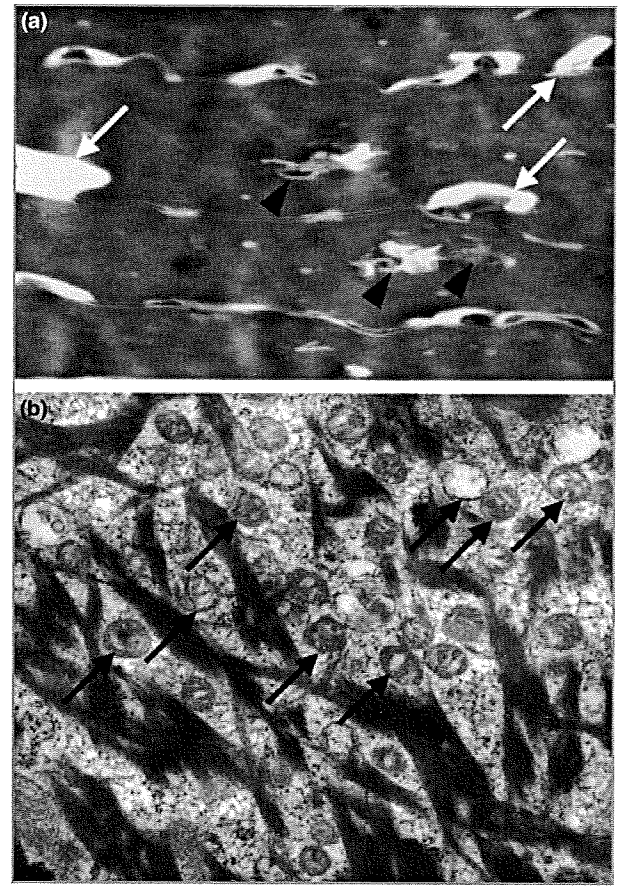

Fig 2. (a) Electron microscopy revealed irregularly dilated intercellular spaces (white arrows) between the keratinized cells and a small number of membranous remnants (black arrows) within the cytoplasm of keratinized cells. (b) In a granular layer cell, lamellar granules lacking lamellar structure, vacant or containing irregular sized vesicles (black arrows) were observed. Original magnification: (a) × 12 000, (b) × 60 000.

In 1984, Kolde and Happle[10] reported morphological changes in the lesional skin of patients with CHH. In their report, numerous small to medium-sized vacuoles measuring between 0·4 and 1·5 μm in diameter were observed in the granular layer keratinocytes, as seen in the present study. In 1994, vacuolated lamellar granules and a lack of intercellular lamellar structures were shown in an infant with CHH,[5] although the causative molecule or gene was not elucidated in those studies. In this report, for the first time, in a patient with CHH with a confirmed EBP mutation, we demonstrated abnormal lamellar granule contents in the granular layer cells in the lesional epidermis. The present ultrastructural findings suggest that EBP mutations and consequent cholesterol deficiency lead to defective lamellar granule contents, resulting in malformed intercellular lipid layers and the ichthyotic skin phenotype characteristic of patients with CHH.

Kolde and Happle[11] reported Langerhans cell degeneration and reduced density of Langerhans cells in the patients' epidermis and suggested that the ichthyotic phenotype of CHH is caused by Langerhans cell depletion. However, in the present study, no significant reduction of Langerhans cell density was observed in the patient's epidermis.

Kolde and Happle[10] reported that hair follicles showed signs of atrophy which was in an early stage in a 4-week-old baby, and was fully developed in a 14-year-old girl, although the other morphological abnormalities were similarly observed in both the baby and the 14-year-old girl. In the present study, no apparent atrophy of hair follicles was seen, probably because the skin biopsy sample was taken in the neonatal period.

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Key words: cholesterol, EBP, ichthyosis, lipid

Conflicts of interest: none declared.

Acquired idiopathic generalized anhidrosis: possible pathogenic role of mast cells

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Sir, A 37-year-old man experienced anhidrosis of almost his entire body and cholinergic urticaria accompanied by severe heat intolerance for 5 months, which caused him to leave his job as an electrical engineer. A physical examination revealed no abnormalities except for slightly dry skin on his trunk and extremities. A neurological examination yielded no abnormal findings for his sensory system and sympathetic function. Laboratory tests, including blood counts, antinuclear antibody, anti-SS-A/Ro, anti-SS-B/La, total IgE and other biochemical profiles were normal except for a slightly elevated total bilirubin (1·2 mg dL^{-1}; normal 0·0–1·0 mg dL^{-1}).

Intradermal injection of 0·05 mL acetylcholine (100 μg mL^{-1}) produced no local sweating (Fig. 1). A thermoregulatory sweating test using the iodine–starch method showed almost generalized anhidrosis except for the axillary zones (Fig. 1). After 15 min of exercise on a treadmill, only 0·06 mL of sweat was collected from both forearms; pinpoint-sized weals characteristic of cholinergic urticaria were observed. In order to check his responsiveness to autologous sweat, autologous sterilized sweat (diluted 1 : 100) was injected intradermally, resulting in a negative response.[1] A skin biopsy specimen was taken from his right forearm where sweating did not occur. The eccrine glands and ducts were surrounded by infiltrates of CD3-positive lymphocytes and a considerable number of mast cells (0·88 mast cells per gland) that were metachromatically stained with toluidine blue (Fig. 2). Serial sections of the skin biopsy revealed focal hyperkeratosis at the acrosyringium and normal eccrine glands (Fig. 2).

The patient was diagnosed with acquired idiopathic generalized anhidrosis (AIGA) accompanied by cholinergic urticaria. Firstly, loratadine 10 mg daily was administered but this treatment was not effective for the cholinergic urticaria or anhidrosis. Next, methylprednisolone 1000 mg daily was

COMMENT AND RESPONSE

Redundant Data in the Meta-analysis on *Helicobacter pylori* Eradication

TO THE EDITOR: We read with interest the recent meta-analysis by Fuccio and colleagues (1). There is an ongoing Cochrane review on this subject; its protocol is published (2), and its results have been reported in abstract form (3). Fuccio and colleagues report that eradication therapy for *Helicobacter pylori* reduces the subsequent incidence of gastric cancer. However, we have serious doubts about the accuracy of their analysis. In the Methods section of their article, Fuccio and colleagues state that when they found multiple articles for a single study, they used the latest publication from each eligible study. However, the meta-analysis erroneously incorporated data from the same randomized, controlled trial (RCT) twice (4, 5). Tables 1 and 2 (1) showed "2" studies that were conducted in the Shandong province of China, commenced in 1996, had very similar numbers of participants, and used identical eradication regimens of the same duration. We contacted the original investigators of these studies (2, 3) directly when collecting data for our review. Those investigators confirmed that the 2 publications were in fact 5-year (4) and 10-year (5) follow-up studies from the same RCT.

The erroneous inclusion of repeated data led Fuccio and colleagues to report a statistically significant effect of *H. pylori* eradication therapy in reducing incidence of gastric cancer, when this is not the case. If only the 10-year follow-up data are included (5), the pooled relative risk for subsequent gastric cancer is 0.65 (95% CI, 0.42 to 1.01). Also, the pertinent trial publications reported fewer cases of gastric cancer after 10-year follow-up (5) than at 5-year follow-up (4), raising concerns about the accuracy of data collection or of reporting for the trial. Taken together, this information convinces us that the effect of eradication therapy on gastric cancer is not as clear-cut as Fuccio and colleagues suggest.

We delayed publishing our full Cochrane review because of these concerns about the duplicate Chinese studies. Our knowledge and experience regarding errors in meta-analyses and the fact that *Annals* published the meta-analysis with erroneous data highlight several important issues for authors of meta-analyses, for journal editors, and for peer reviewers. First, transparent reporting of follow-up studies conducted at various time points from the same RCT is needed to ensure correct identification of trials and to avoid miscounting of data by authors. Second, when studies are reported only in abstract form, or if the accuracy of the data is in doubt, authors should directly contact original investigators for clarifications. Finally, peer reviewers (and journal editors) may have difficulty confirming the results of systematic reviews and meta-analyses. In some cases, independent verification of the meta-analysis may be needed to ensure the results are truly accurate.

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IN RESPONSE: Drs. Ford and Moayyedi write that we counted the same trial twice in our recently published meta-analysis. Reports of the "2" pertinent trials (1, 2) were not written by the same authors. The 2 reports showed that the work had been carried out in the Shandong province of China in the 1990s and used the most common worldwide eradication treatment at that time (1 week of omeprazole, clarithromycin, and amoxicillin). The 2 reports, as well as a third related report, showed confusing discrepancies regarding recruitment methods for participants and numbers of patients randomly assigned to eradication treatment and control groups (1–3). These reports also gave discrepant information on eradication rate of *H. pylori* in the treated group, baseline histologic characteristics of included patients, histologic classification system used, age inclusion range and mean age of included patients, and number of gastric cancer cases in each group. Therefore, we thought the 2 studies were different, although we did try to contact authors for clarification and confirmation. Our attempt to contact Leung and colleagues (1) by e-mail was unsuccessful. Zhou responded to our request for additional information by sending us 3 publications (2–4), but made no reference to the study by Leung and colleagues.

We thank Drs. Ford and Moayyedi for pointing out that Zhou's report (2) may represent 10-year follow-up data from the same trial for which Leung and colleagues reported 5-year follow-up data (1). However, if this is so, how can there be fewer cases of gastric cancer in the treatment group at 10 years ($n = 2$) than at 5 years ($n = 4$)? It calls the accuracy of these data into serious doubt, and one must ask which of the 2 groups of data, if either, should be considered. We strongly agree with Drs. Ford and Moayyedi about the need for transparent reporting for follow-up studies conducted at different stages of the same RCT.

Finally, we avoided emphasizing the statistical significance of the results of our meta-analysis because the few available studies did not allow firm conclusions. A relative risk of 0.65 (CI, 0.43 to 0.98; $P = 0.04$) is very close to that obtained after excluding the 5-year follow-up data reported by Leung and colleagues (1): relative risk, 0.65 (CI, 0.42 to 1.01; $P = 0.05$). Clinical and statistical significance do not always correspond. Therefore, even after excluding the study by Leung and colleagues, our conclusions remain the same: *Helicobacter pylori* eradication treatment seems to modestly reduce the risk for gastric cancer. However, from a clinical point of view, even a small reduction in risk and incidence achieved with *H. pylori*

eradication treatment will probably give a huge advantage in terms of social health, especially in high-risk areas.

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Potential Conflicts of Interest: None disclosed.

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CLINICAL OBSERVATIONS

Granulysin as a Marker for Early Diagnosis of the Stevens–Johnson Syndrome

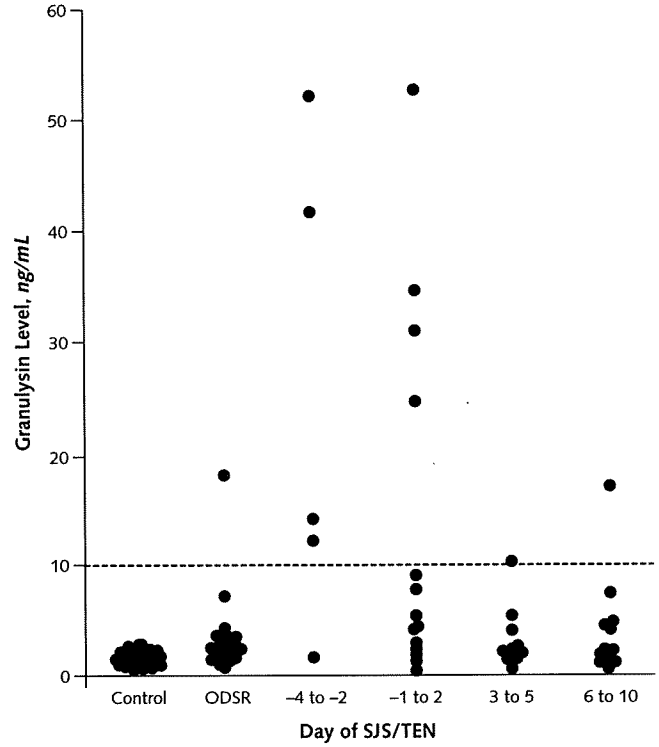
Background: The Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by massive epidermal necrosis. In the early stage, clinical presentations of SJS/TEN are very similar to those of ordinary drug-induced skin reactions (ODSRs); therefore, SJS/TEN is difficult to diagnose and the start of treatment is often delayed, resulting in high mortality rates. Other investigators (1) reported that granulysin is highly expressed in blisters of SJS/TEN and causes disseminated keratinocyte death. Because SJS/TEN progresses and spreads rapidly, the granulysin level should be increased in the serum of patients with active SJS/TEN if it is a key mediator of these diseases.

Objective: To determine whether serum granulysin levels are higher in patients with SJS/TEN than in healthy control participants or those with ODSRs.

Methods: We measured granulysin in the sera of 31 healthy control participants, 24 patients with ODSR, 13 patients with SJS, and 7 patients with TEN by using enzyme-linked immunosorbent assay (2). Disease onset in patients with SJS/TEN was defined as the day (day 1) on which the mucocutaneous or ocular lesion first eroded or ulcerated (3), and we collected sera from these patients from 4 days before to 10 days after ulceration. We used the Tukey–Kramer test to conduct multiple comparisons between groups.

Results: None of the 31 healthy control participants had a granulysin level greater than the upper limit of normal, which was 10 ng/mL (0% elevated; mean, 1.6 ng/mL [SD, 0.6]), and among 24 patients with ODSRs, only 1 patient had an elevated granulysin level (4.2% elevated; mean, 3.5 ng/mL [SD, 3.4]) (**Figure**). We obtained samples from 5 patients with SJS/TEN on day −4 to day −2, and we detected the highest granulysin concentrations (elevated in 80% of patients); mean, 24.8 ng/mL [SD, 21.2]). Granulysin levels were lower in the 14 samples collected on day −1 to day 2 (28.6% elevated; mean, 13.7 ng/mL [SD, 16.0]), and were even lower in the 10 samples collected from day 3 to day 5 (10.0% elevated; mean, 4.2 ng/mL [SD, 3.0]) and in the 13 samples collected from day 6 to day 10 (7.7% elevated; mean, 4.5 ng/mL [SD, 4.5]). When we compared granulysin levels from day −4 to day −2 among patients with SJS/TEN, patients with ODSRs, and healthy control participants, the differences were statistically significant ($P < 0.010$).

Figure. **Granulysin levels of healthy control participants, patients with ODSRs, and patients with SJS/TEN.**



ODSR = ordinary drug-induced skin reaction; SJS/TEN = Stevens–Johnson syndrome/toxic epidermal necrolysis.

Discussion: Granulysin is cytotoxic for tumor cells, transplant cells, bacteria, fungi, and parasites, in which it damages negatively charged cell membranes because of its positive charge (4). It plays an important role in the host defense against pathogens, and it induces apoptosis of target cells by using a mechanism involving caspases and other pathways (4). Its potency makes it a credible mediator of skin damage in patients with SJS/TEN. Adding to this credibility is a report (1) that granulysin is the most highly expressed cytotoxic molecule in the blisters of patients with SJS/TEN. We show that serum granulysin levels in 4 of 5 patients with SJS/TEN were elevated before skin detachment or mucosal lesions develop. Soluble Fas ligand (sFasL) shares some properties with granulysin: It contributes to keratinocyte death in SJS/TEN (3, 5), and levels are elevated in the sera of patients with SJS/TEN (3). Serum granulysin levels, however, are approximately 100 times higher than those of sFasL on day

−4 to day −2 (23.1 ng/mL [SD, 16.6] vs. 147.76 pg/mL [SD, 104.4]). Therefore, we believe it would be easier to develop bedside granulysin serum measurement, for example, by using immunochromatography, than it would be to develop a similar sFasL measurement. Monitoring serum granulysin might enable early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that otherwise could not be distinguished from ODSRs.

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Localized Amyloidosis at the Site of Enfuvirtide Injection

Background: Enfuvirtide is the first of a new class of antiretroviral agents that block fusion of the viral particle with the host target cell. Its safety and antiviral activity have been demonstrated (1, 2). In clinical trials, injection site reactions occurred in 80% to 100% of patients (3). The most common signs and symptoms reported were induration in 94%, erythema in 91%, and subcutaneous nodules and cysts in 70% (4).

Objective: To describe a case of amyloidosis at the injection site of enfuvirtide.

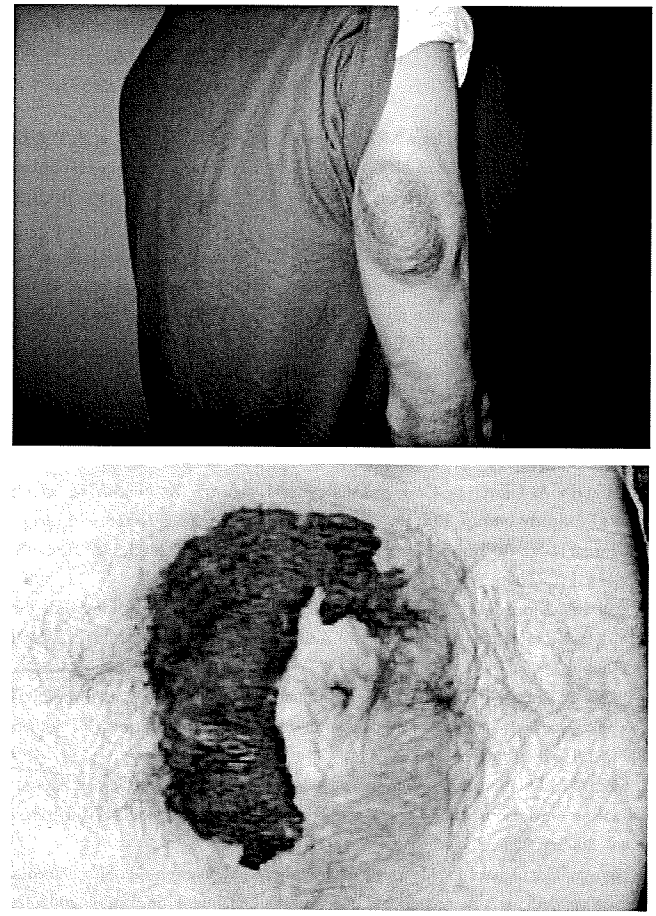
Case Report: The patient was a man aged 47 years who had a history of sexual intercourse with men and extensive treatment for HIV with a triple-class viral resistance profile. He also had long-standing leg pain thought to be secondary to HIV neuropathy and no history of intravenous drug use. There was no history of opportunistic or chronic infections.

Because of a persistently elevated viral load, enfuvirtide by subcutaneous injection was added to his highly active antiretroviral treatment regimen for 41 months; enfuvirtide therapy was then stopped in February 2007 because of intolerable injection site reactions. While he was receiving enfuvirtide, his viral loads were completely suppressed. Eighteen months after enfuvirtide therapy was stopped, large, tender, indurated reactions with fragile epithelial surfaces persisted at all injection sites (**Figure**, *top*). These reactions bled extensively into the subcutaneous tissue with minor trauma (**Figure**, *bottom*). A lesion on the triceps was excised surgically, and the wound healed without complications. Pathologic examination showed extensive deposits of proteinaceous material with intense Congo red staining that was consistent with amyloid. A lesion on the opposite arm was resected and showed similar findings. The patient had a normal leukocyte count and normal hemoglobin, blood urea nitrogen, and creatinine levels and had no evidence of plasma cell dyscrasia and no history of organ dysfunction to suggest systemic amyloidosis.

Discussion: In 7 patients receiving enfuvirtide, biopsy of injection site reactions revealed an inflammatory response consistent with a localized hypersensitivity reaction (5), and other studies (3) have reported similar findings. Other reports (6) have described 3 histologic patterns: an acute urticaria- or vasculitis-like pattern with inflammation of the fat tissue, a subacute pattern with an initial dermal sclerosis, and a long-term scleroderma-like pattern.

In our patient, surgical excision of enfuvirtide injection site reactions revealed subcutaneous nodular amyloidosis. Localized

Figure. **Lesion in right triceps area (*top*) and periumbilical site with spontaneous intradermal and subcutaneous hemorrhage (*bottom*).**



subcutaneous nodular amyloidosis is rare (7, 8). The lesions can present as waxy nodules with or without overlying atrophic epidermis, and they may ulcerate with minimal trauma, causing cutaneous hemorrhage (7–9). Some authors (7) have reported that nodular cutaneous amyloidosis may occur in relation to cutaneous plasmocytoma.

Amyloid formation at the site of drug injection has been described in 5 previous patients. Of these, 4 were receiving either porcine or human insulin for glucose control (10–13), and 1 was given injections of an unknown medication during the Korean War (14). To our knowledge, this is the first case of localized amyloidosis associated with the use of enfuvirtide. We believe that localized amyloidosis should be considered in patients with severe, persistent injection site reactions and suggest that subcutaneous hemorrhage may help make the diagnosis.

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CORRECTIONS

Correction: Can *Helicobacter pylori* Eradication Treatment Reduce the Risk for Gastric Cancer?

A recent article (1) that pooled data from 6 trials reported that, compared with no treatment, *Helicobacter pylori* eradication treatment reduced the relative risk (RR) for gastric cancer (0.65 [95% CI, 0.43 to 0.98]). The article described and counted data from "2" trials that the editors believe were actually 5- and 10-year follow-up data from the same trial (2, 3). Furthermore, the reported data for the eradication treatment group for the trial showed fewer cases of gastric cancer at 10-year follow-up ($n = 2$) than at 5-year follow-up ($n = 4$). Reported numbers for the 10-year follow-up were extracted from an abstract presentation (3). If the reported data from the 5- or 10-year follow-up are excluded, the pooled RR is 0.65 (CI, 0.42 to 1.01) or 0.70 (CI, 0.46 to 1.08), respectively. If reported data from both the 5-year and 10-year follow-up are excluded, the pooled RR is 0.71 (CI, 0.45 to 1.23).

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Correction: Predicting Deep Venous Thrombosis in Pregnancy

There are 2 errors in a recent article on prediction of deep venous thrombosis in pregnancy (1). In Table 5, under "LEFt variables," the second subheading should say "≤1 or >1," and the first line under that should say "≤1." The online version has been corrected.

Reference

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Letter to the Editor

Four novel mutations of the *ADAR1* gene in dyschromatosis symmetrica hereditaria

Dyschromatosis symmetrica hereditaria (DSH: MIM#127400) is a highly penetrant autosomal dominant skin disease characterized by hyperpigmented and hypopigmented macules on the face and dorsal aspects of the extremities. This disorder commonly develops during infancy or early childhood [1]. Previous work has shown that a heterozygous mutation of the adenosine deaminase acting on RNA 1 gene (*ADAR1* or *DSRAD*) causes DSH in four Japanese DSH families [2]. Subsequently, more than 60 mutations in the *ADAR1* gene have been reported in Japanese, Chinese, and Taiwanese patients with DSH [3–8]. These data confirm that mutations in the *ADAR1* gene are responsible for DSH in Japanese and other ethnic groups. The ADAR1 protein catalyzes the deamination of adenosine to inosine in double-stranded RNAs [9]. This creates alternative splice sites or alternations of the codon which leads to functional changes in the target substrate(s). However, the target gene(s) for ADAR1 in the skin as well as the mechanisms by which mutations in *ADAR1* cause DSH remain unknown. In this study, we report four novel mutations of the *ADAR1* gene in Japanese patients with DSH.

We have identified four novel heterozygous *ADAR1* mutations including two missense mutations (p.S915F, p.S1154F), one nonsense mutation (p.Y989X), and one splice site mutation (IVS4-2A→G) in three families and one sporadic patient (Table 1). The mutational analysis of the *ADAR1* gene was performed as previously described [3]. All patients were Japanese and unrelated. Patient 2 had no family history of DSH. All other patients had one or more affected family members. We screened at least one affected individual of each pedigree for mutations in the *ADAR1* gene. The patients originally consulted us for their skin conditions. All patients phenotypically presented typical macules on the dorsal aspects of the hands, feet, lower arms and lower legs, and freckle-like macules on the face. Informed consent and blood samples of patients were obtained under protocols approved by the Ethics Committee of Yamagata University School of Medicine.

Sequencing from amplified DNA from affected individuals revealed two novel missense mutations (p.S915F, p.S1154F) that altered amino acids conserved among all known species, including pufferfish, zebrafish, frog, rat, mouse, cow, and human within the deaminase domain of ADAR1. These mutations were not detected in the control blood samples of the surveyed 107 unrelated, normally pigmented Japanese adults. Therefore, we consider these mutations pathologic with no functional activity. To date, 30 missense mutations, including our identified 2 missense mutations, have been reported. Interestingly, all known missense mutations are located within the deaminase domain, which encompasses amino acids 886–1221, suggesting that this domain is critical for enzyme function.

In the case of the splice site mutation detected in Patient 3, IVS4-2A→G, we tried to confirm an aberrant splice product by RT-PCR. However, we failed to find mutant mRNA derived from the mutant allele, suggesting that nonsense-mediated mRNA decay (NMD) may have degraded the mutant transcript. Therefore, we quantified by real-time quantitative RT-PCR (RQ-RCR) the amount of *ADAR1* transcript in affected individuals from RNA in peripheral blood. The *ADAR1* gene was previously confirmed to express ubiquitously including in skin and peripheral blood [10]. RQ-PCR was performed as previously reported [5] with some modification. An amplicon of 112 bp in length was obtained with the primer set, E11QF 5′-AGCTCCGCACCAAGGTGGAGAAC-3′ and E12GR 5′-CAGGACATGGTACGGAGTCTC-3′ and normalized with a beta-2-microglobin (B2M) fragment. Reactions were performed using the STRATAGENE Mx3000P Real-Time QPCR System under relative quantification with Brilliant II SYBR Green QPCR Master Mix® (STRATAGENE, La Jolla, CA). Data were analyzed with MxPro™ Software ver 4.0 (STRATAGENE, La Jolla, CA). As shown in Fig. 1, approximately 50% of *ADAR1* mRNA was reduced in cells from Patient 3 compared to two healthy volunteers. Also no mRNA reduction was observed in Patient 4, who had a missense mutation (p.S915F), indicating that the aberrant transcript derived from the IVS4-2A→G allele, in Patient 3, may have been degraded by an NMD mechanism, resulting in *ADAR1* haploinsufficiency. This result supports previous reports that DSH may be caused by *ADAR1* haploinsufficiency [5]. It was not determined whether NMD occurred in Patient 1, who had a nonsense mutation (p.Y989X), since this patient did not agree to provide additional blood samples for RQ-PCR.

In conclusion, we have found four novel mutations in the *ADAR1* gene of three DSH pedigrees and one sporadic individual. Of the mutations, a splice mutation, IVS4-2A→G, was confirmed to be a pathologic mutation leading to NMD and *ADAR1* haploinsufficiency. These results may provide new insight into the pathogenesis of DSH.

1. Electronic database information

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/(for the cDNA of human *ADAR1* [accession number NM_001111.3]).

Acknowledgments

We are grateful to the patients, their families and volunteers for providing blood samples. This work was supported by grant 18659322 (T. Suzuki) and 19390294 (Y. Tomita) from the Ministry of Education, Science and Culture of Japan.

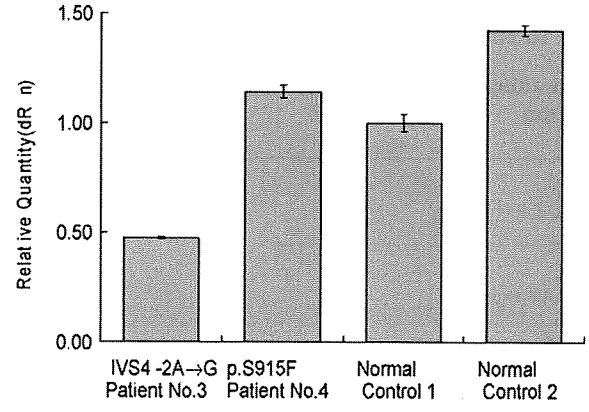

Fig. 1. Relative quantity of *ADAR1* transcripts compared normal control 1. Data derived from real-time quantitative RT-PCR are expressed as mean ± S.D. of three independent experiments performed in triplicate.

☆ This work was done in Yamagata, Japan.

Abbreviations: DSH, dyschromatosis symmetrica hereditaria; ADAR1, adenosine deaminase acting on RNA 1; RT-PCR, reverse transcription-polymerase chain reaction; RQ-RCR, real-time quantitative RT-PCR.

Table 1
Novel mutations of the *ADAR1* gene

Patient No.[a]	Patient's pedigree			Mutation			Phenotype		Polymorphism
	Incidence	Affected individuals	Unaffected individuals	Nucleotide change[b]	Amino-acid change	Position	Onset	Degree	
1	Familial	2	3	c.C2967A	p.Y989X	Exon 11	3 years	Strong	
2	Sporadic	1	–	c.C3461T	p.S1154F	Exon 15	3 years	Strong	
3	Familial	8	7	IVS4-2A→G	–	Intron 4	1–2 years	Strong	c.1752–1754delATC, p.S585del in Exon 3
4	Familial	10	8	c.C2744T	p.S915F	Exon 9	5 years	Faint	c.A1151G, p.K384R in Exon 2

[a] A serial number of patients in our group.
[b] GenBank accession no. NM_001111.3. Position 1 is A of the translation initiation codon.

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Letter to the Editor

Keratin alterations could be an early event of wrinkle formation

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Long-term alteration of keratins
Fine wrinkle

Abbreviation: KIFs, keratin intermediate filaments.

Wrinkle formation is a common sign of photo aging. There have been many studies on formation of wrinkles. Among the several proposed mechanisms, it is widely accepted that formation of wrinkles, induced by chronic solar exposure, is closely linked to the loss of elastic properties of the skin and that denature of collagen and elastic fibers reduce skin elasticity [1].

In addition, there are many reports suggest that stratum corneum plays an important role on fine wrinkle formation. The lack of water-holding capacity of the stratum corneum or skin dryness can also cause fine wrinkles. Moreover, the water content, the water-holding capacity and the hygroscopicity of the stratum corneum decreased after ultraviolet B (UVB) exposure [2].

Besides these proposals, we aimed at keratins in epidermis for sustaining skin elasticity. Keratins are the major structural proteins of vertebrate epidermis, constituting up to 85% of fully

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Mucinous eccrine naevus

Editor

Mucinous eccrine naevus is a very rare variant of eccrine naevus with only five cases previously reported.[1–5] Histopathological characteristics are proliferation of normally structured eccrine glands surrounded by abundant mucin deposits. We describe a case of mucinous eccrine naevus, which presented as swollen patches.

A 4-month-old Japanese girl presented with a 1-month history of multiple lesions over the extensor surface of her right forearm. Physical examination revealed more than fifteen irregular nodules along the right forearm that were 1–1.5 cm in diameter (Fig. 1a). The nodules were brownish in part and firm to palpation with much soft hair. The lesions were asymptomatic and hyperhidorosis was not evident. Clinical examination was otherwise unremarkable, and her development was normal. There was no history of antecedent trauma to the area or family history of similar lesions. At this stage, a clinical diagnosis of mastocytoma was suspected; a naevus of sweat gland or smooth muscle.

We performed a skin biopsy from the brownish nodule after informed consent was obtained from the parents. Histologically, the epidermis showed mild hyperkeratosis and acanthosis with no remarkable changes evident in the superficial dermis. In contrast, the middle to deep dermis was markedly abnormal. Abnormalities included proliferation of lobulated eccrine glands, an increase in the number of intradermal eccrine ducts and eccrine coils composed of a normal secretory and ductal portion with abundant mucinous material within the surrounding stroma (Fig. 1b). This deposit was identified as mucin as it was stained by pH 2.5 Alcian blue staining and exhibited metachromatism following toluidine blue staining. Mild fibroblastic hyperplasia was present in the dermis, but there was no angiomatous component. Immunostaining for desmin and actin was negative except for the erector pili muscles. As a result of these clinical and histopathological findings, a diagnosis of mucinous eccrine naevus was made.

At the 2-month follow-up assessment, the lesions were tender and exhibited increased swelling accompanied by erythema. The application of topical steroids relieved the discomfort, but the swelling of the lesions did not completely resolve.

Eccrine naevus is a rare hamartoma and is histologically characterized by the size and number of normal eccrine glands. Eccrine naevus presents in childhood and adolescence and approximately half of the lesions occurred on the forearms. Hyperhidorosis was found in some cases.[6] Mucinous eccrine naevus is a variant of eccrine naevus. Romer and Taira[1] used this name for the first time in 1994. Thereafter, five cases have been reported.[1–5] The characteristic histopathological finding that is key for the diagnosis is the proliferation of normally structured eccrine glands surrounded by abundant mucinous material in the dermis. The lesions appeared in childhood in four cases including our case, whilst two cases presented in middle age. The clinical manifestation was firm, erythematous, brownish patches or nodules that tended to be accompanied by tender spots. The nodules were around 1–2 cm in size with the largest case[5] being approximately 4 cm. Espana *et al.* reported a unique case that showed a distribution following the lines of Blaschko.[4] Plastic surgery was required to deal with the hypertrophy in a case, but no treatment was required in the other cases. In our case, a topical steroid ointment was used for the tender spots with some beneficial effect.

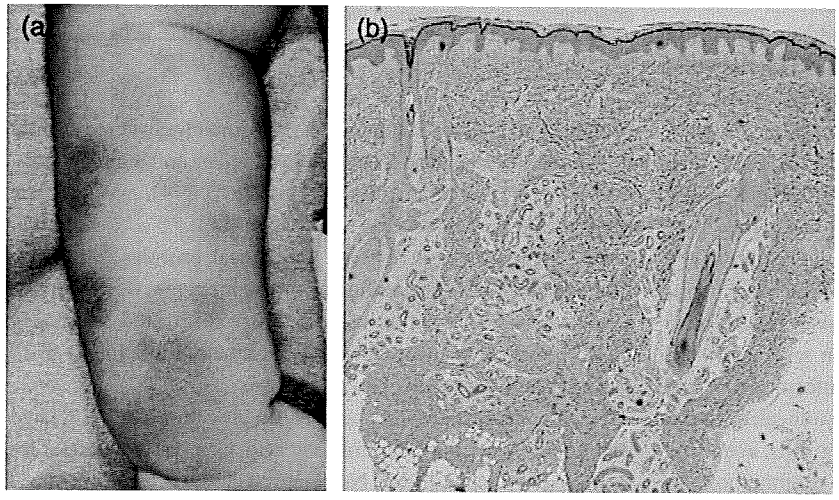

Figure 1 (a) Multiple erythematous brownish irregular nodules on the right forearm. (b) A skin biopsy showed an increased number of eccrine structures that were surrounded by abundant mucinous material in the middle to deep dermis (haematoxylin and eosin; original magnification ×20).

The pathogenesis of mucinous eccrine naevus is currently unclear. Llombart *et al.*[2] suggested that a potential defect in embryogenesis might be involved but also recognized that cases presenting in adulthood argued against this hypothesis. Regarding the mucinous deposit, España *et al.* hypothesized that stimuli such as growth factors that stimulate fibroblasts might play a role in increasing mucin synthesis.[4]

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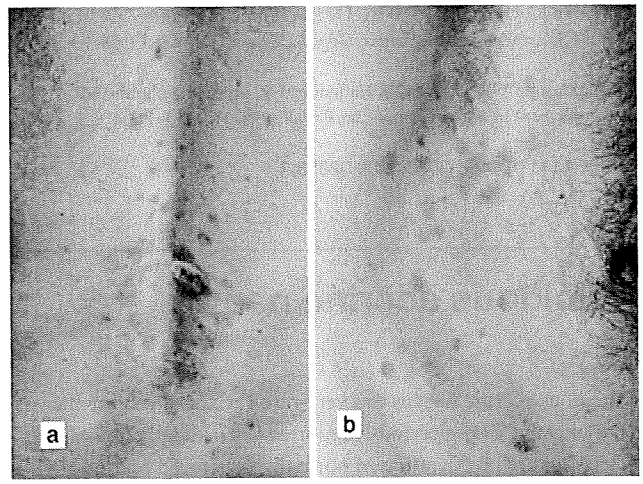

Figure 1 (a) Slightly vesiculous erythematous plaques clustered around a lumbar scar. First waves of lesions were confined to this location, while incisional wound was still unhealed. (b) Similar cutaneous lesions on axillar folds.

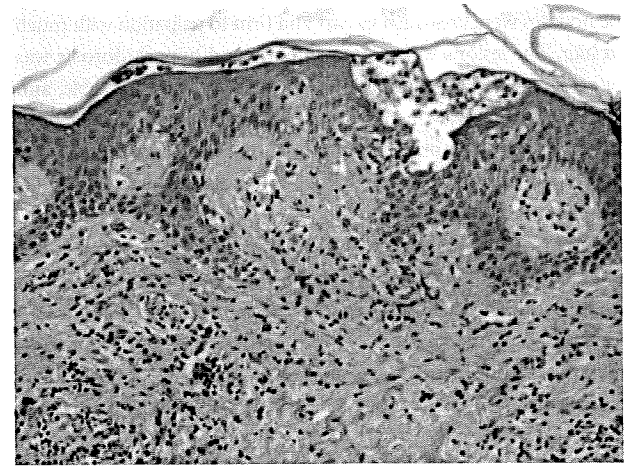

Figure 2 Subcorneal pustule filled with neutrophils. Neither acantholysis nor spongiosis were observed.

Subcorneal pustular dermatosis with pathergy phenomenon in a patient with Crohn's disease

Editor

Subcorneal pustular dermatosis (SPD), or Sneddon–Wilkinson disease, is a chronic relapsing vesiculopustular eruption that mainly affects the trunk and flexures. Its aetiology is still unknown, and it has been considered a neutrophilic disorder, although its differential diagnoses with pustular psoriasis and, more recently, with immunoglobulin A (IgA) pemphigus[1] is still controversial. We report a case of a 44-year-old man, with a large history of Crohn's disease, well controlled and not requiring any treatment at that moment. He showed a 3-month erythematous vesiculopustular relapsing eruption on the lumbar area. A basal cell carcinoma had been excised on that region several months before, suffering a torpid healing. The patient informed that first waves of skin lesions were confined around the unhealed wound and extended afterwards to trunk, axillae and groins, acquiring the typical distribution of SPD. On physical examination, he presented no scaly erythematous plaques, mainly clustered around the lumbar scar, but also affecting chest, axillae and inguinal folds, with scarce vesiculopustules on surface (Fig. 1). No mucosal involvement was observed.

The histopathology revealed subcorneal pustules filled with neutrophils. Neither acantholysis nor spongiosis were present. Upper dermis showed an inflammatory infiltrated consisting of lymphocytes, neutrophils and occasional eosinophils (Fig. 2). Direct immunofluorescence was negative.

Haematological and biochemical investigations only revealed low levels of vitamin B12 and folic acid, as a consequence of his inflammatory bowel disease. No paraprotein was detected.

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LETTER TO THE EDITOR

Functional analysis of OCA4 mutant sequences using *under white* mouse melanocytes

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Dear Sir,

Oculocutaneous albinism (OCA) is a group of autosomal recessive hypopigmentary disorders of the skin, hair and eyes. The four types of OCA (OCA1–4) are the result of single gene mutations in tyrosinase (*TYR*; OCA1 [MIM 203100]), P (*P*; *OCA2* [MIM 203200]), tyrosinase-related protein 1 (*TYRP1*; OCA3 [MIM 203290]) and Solute Carrier Family 45, member 2 (*SLC45A2*; OCA4 [MIM 606574]). The *SLC45A2* gene (formerly membrane-associated transporter protein; MATP) encodes a melanosomal protein, the function of which remains unknown (Newton et al., 2001). Its homolog in medaka fish, *b*, encodes a transporter that mediates melanin synthesis and exhibits structural homology to plant sucrose–proton symporters (Fukamachi et al., 2001). OCA4 is a major OCA type in the Japanese population (~30%), but is rare in Caucasians (Inagaki et al.,2004; Newton et al., 2001; Suzuki and Tomita, 2008). The clinical phenotype of OCA4 patients varies depending on their mutant genotypes. While some resemble OCA1A (tyrosinase-negative OCA) cases and have no pigmentation, others exhibit some generalized pigmentation with brown hair and irides without nystagmus. Moreover, some patients show improvement during the first decade of life (Inagaki et al., 2004; Rundshagen et al., 2004). The human SLC45A2 protein is a 530-amino acid polypeptide that contains 12 putative transmembrane domains, and is only expressed in melanocytes (Newton et al., 2001).

More than thirty-five different mutations have been reported in Turkish, German, Korean, Japanese, Indian, French, Dutch and Belgian OCA4 patients (Inagaki et al., 2004, 2006; Newton et al., 2001; Rooryck et al., 2008; Rundshagen et al., 2004; Sengupta et al., 2007; Suzuki et al., 2005). Two of these mutations, the p.Asp157Asn (p.D157N) and p.Gly188Val (p.G188V) alleles, were the first and second most frequently found variants in Japanese patients, with allele frequencies of 0.39 (14/36) and 0.19 (7/36), respectively (Inagaki et al., 2004). Judging from the relationship between patient phenotypes and genotypes, we previously reported that the p.D157N variant might be non-functional, or have very low functional activity, in melanogenesis. There is also evidence that the p.G188V allele might produce a protein with low functional activity (Inagaki et al., 2004). The function of mutant SLC45A2 proteins was evaluated by comparing the ability of the wild-type versus the mutant polypeptide to produce melanin in melanocytes (*under white* cells; *uw*) established from an OCA4 mouse model (Costin et al., 2003). We report here the differential complementation of defective melanin biosynthesis and the hypopigmentation of *uw* cells by transfection with wild and mutant human *SLC45A2* cDNA sequences.

We constructed wild-type and mutant *SLC45A2* (p.D157N and p.G188V) cDNA constructs. Full-length wild-type human *SLC45A2* cDNA was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA). Site-directed mutagenesis was used to introduce substitutions for the G at nucleotide positions (n.p.) 469 and 563 with A in p.D157N and T in p.G188V. The cDNA products were sequenced to confirm accuracy and inserted into mammalian expression plasmid pIREShyg3 (BD Biosciences, San Jose, CA, USA), which contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), creating pIREShyg3-SLC45A2 wild, pIREShyg3-SLC45A2mutant-p.D157N and pIREShyg3-SLC45A2mutant-p.G188V. Following an initial 24-hr culture period, *uw* melanocytes were transfected with 1.6 μg of one of the three constructs or pIREShyg3 for mock-transfected cells (per 4cm^2 flask) using Lipofectamine 2000 Reagent (Invitrogen). Stable transformants were selected in culture media containing 500 μg/ml of hygromycin B (Invitrogen). Six independent clones established from each transformant were harvested separately from each plate by trypsinization. Expression of *SLC45A2* mRNA in each of the six clones was confirmed with RT-PCR (Figure 1). For melanin assays, each suspension was pelleted and incubated at 95°C for 1 h after resuspension in 100 μl of 1 N NaOH. After a 100× dilution, the OD_{475} was measured and